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(54) Electrotransfer of nucleic acid into tissue cells

(57) The electrotransfer of a nucleic acid into muscle cells is carried out by an electric stimulation of the tissue as follows:
- first with at least one, preferably a single, pulse of a High Voltage field strength of between 200 and 2000 volts/cm
- second with a single pulse of Low Voltage field strength of between 50 and 140 volts/cm and of duration of between 300 ms and 2000 ms.

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Description

[0001] The present invention is related to the electrically mediated gene transfer of nucleic acids into tissue cells, in particular muscular cells.

[0002] Electrically mediated gene transfer, also termed DNA electrotransfer or electrogenetherapy, has gained a real interest as it is one of the most effective methods of *in vivo* non-viral gene transfer (Andre and Mir, 2004). The method has been shown to be effective to electrotransfer plasmid DNA to various tissues: muscles (Aihara and Miyazaki, 1998; Mir *et al.,* 1998a; Mir *et al.,* 1999), liver (Heller *et al.,* 1996; Suzuki *et al.,* 1998), skin (Titomirov *et al.,* 1991; Zhang *et al.,* 1996), tumors (Heller *et al.,* 2000; Wells *et al.,* 2000; Heller and Coppola, 2002), mouse testis (Muramatsu *et al.,* 1997; Muramatsu *et al.,* 1998), etc (Andre and Mir, 2004).

[0003] The mechanisms by which electric pulses mediate DNA transfer into target cells are not well understood. Nevertheless, there is a common agreement that for an improved DNA transfer into tissues, cells in that tissue must be permeabilized. Such a permeabilization can be achieved using simple runs of short square wave electric pulses (in the range of 100 μs) (Mir *et al.,* 1991b; Gehl *et al.,* 1999; Miklavcic *et al.,* 2000). This kind of pulses has been widely used for the local delivery of non-permanent anticancer drugs (like bleomycin or cisplatin) in a treatment termed 'antitumor electrochemotherapy' (Mir *et al.,* 1991a; Glass *et al.,* 1997; Sersa *et al.,* 1998; Mir *et al.,* 1998b; Rodriguez *et al.,* 2002). Indeed, the delivery to tumors of e.g. 8 pulses of 1300 V/cm and 100 μs either *in vitro* or *in vivo* is sufficient to induce transient rearrangements of the cell membrane that allow non-permeant anticancer molecules like bleomycin to enter the cell by diffusion and to fully exert their cytotoxic activity (Poddevin *et al.,* 1991; Mir *et al.,* 1991 b; Gehl *et al.,* 1998).

[0004] These short permeabilizing electric pulses have also been shown to increase the transfer of plasmid DNA into several tissues (Heller *et al.,* 1996; Heller *et al.,* 2000). However, another type of square-wave electric pulses was applied to muscles (Aihara and Miyazaki, 1998; Mir *et al.,* 1999), tumors (Rols *et al.,* 1998), liver (Suzuki *et al.,* 1998) and some other tissues (Andre and Mir, 2004), and was found to be more effective for DNA electrotransfer *(Mir et al.,* 1999; Heller *et al.,* 2000). These pulses usually are of lower voltage but much longer duration (in the range of tens of milliseconds) (Aihara and Miyazaki, 1998; Rols *et al.,* 1998; *Mir et al.,* 1999; Bettan *et al.,* 2000; Matsumoto *et al.,* 2001). It is assumed that this type of pulses mediate DNA transfer into the cells by inducing two distinct effects that include cell permeabilization (like the short pulses) and DNA electrophoretic migration during the delivery of the electric field (Klenchin *et al.,* 1991; Sukharev *et al.,* 1992; Neumann et *al.,* 1996; Mir *et al.,* 1999; Golzio *et al.,* 2002).

[0005] Efficient electrotransfer into muscle cells has been described in WO-A-99/01158 using one or more (up to 100,000) unipolar electric impulsions of 1-800 volts/cm and in WO-A-98/43702 using stimulation with an electric current of 5-200 volts/cm, wherein the electric current may be in the form of 2-30,000 square bipolar pulses.

[0006] The double role of the electric pulses on *in vivo* DNA electrotransfer was demonstrated by using combinations of electric pulses consisting of high voltage, short pulses (or HV; e.g. 800 V/cm and 100 μs) followed by low voltage, long pulses (or LV; e.g. 80 V/cm and 100 ms) (Bureau *et al.,* 2000; Satkauskas *et al.,* 2002). In this last study it has been shown that these HV and LV pulses can be separated by various lags between the HV and the LV(s) without significant loss in transfection efficiency. These lags ranged up to 300 s for 1 HV and 1 LV, and up to 3000 s for 1 HV and 4LV combinations (Satkauskas *et al.,* 2002).

[0007] The applicant has found that it was still possible to improve the electrotransfer efficiency by using a specific combination of HV and LV pulses.

[0008] Transfection of tumors and/or other tissues e.g. the liver, can also be of interest for similar purposes. Preferred electric field strength (in V/cm) for the HV and/or the LV will change according to the tissues.

[0009] A first object of the invention is thus the use of a nucleic acid for the preparation of a human or veterinary medicament or drug intended to be transferred *in vivo* into tissue cells, wherein the medicament is brought into contact with tissue cells and the tissue is electrically stimulated as follows:

- first with at least one pulse of a High Voltage (HV) field strength of between 200 and 2000 volts/cm
- second with a single pulse of Low Voltage (LV) field strength of between 50 and 140 volts/cm and of duration of between 300 and 2000 ms.

[0010] A used herein, the term "tissue" denotes a tumoral or non tumoral tissue of an animal, for instance a human, or a non human Mammal such as a rodent (e.g. a mouse, or a rat), a dog, a cat, or a primate. A non tumoral tissue may be a muscle or liver.

[0011] According to an embodiment, the tissue is a muscle. It is preferred that said muscle be electrically stimulated first with at least one pulse of a HV field strength of between 200 and 1400 volts/cm.

[0012] Preferably, the medicament is intended to be brought into contact with the tissue cells before applying the single LV pulse and still more preferably, before the application of the HV pulse or pulses. Typically, the medicament has been brought into contact with the tissue cells from few seconds to 10 minutes, preferably 5 to 10 minutes before the HV pulse or pulses. The medicament may be brought into contact through direct intramuscular injection, through

systemic administration (e.g. intravenous or intra-arterial route) or by topical or subcutaneous administration.

[0013] In an advantageous aspect of the invention, the single LV pulse has a field strength of between 80 and 120 volts/cm, preferably of between 90 and 110 volts/cm, typically about 100 volts/cm.

[0014] In another advantageous aspect of the invention, the single LV pulse has a duration of between 300 and 800 ms, preferably of between 350 and 600 ms, typically about 400 ms.

[0015] The LV pulse may be of the same polarity than the HV pulse.

[0016] However, according to an advantageous aspect, the LV pulse has a polarity opposed to that of the HV pulse.

[0017] Preferably, the single LV pulse is a squared pulse. It can also be trapezoidal, or discontinuous.

[0018] Without being bound to theory, it is deemed the single LV pulse according to the invention at least improves the nucleic acid electrophoretic migration.

[0019] There can be several HV pulses, i.e. from 2 to 10 HV pulses having the specifications disclosed therein. It is more convenient in this case to have identical HV pulses.

[0020] However, it has been demonstrated that a single HV pulse having the specifications disclosed therein is sufficient to permeabilize the cell membrane. Therefore, in the preferred embodiment, use is made of a single HV pulse.

[0021] In a further advantageous aspect of the invention, the HV pulse has a field strength of between 300 and 1300, preferably of between 400 and 1200 volts/cm, more preferably of between 500 and 900, still more preferably of between 600 and 800 volts/cm, typically about 700 volts/cm.

[0022] In still a further advantageous aspect of the invention, the HV pulse has a duration of between 10 and 1000 μs, preferably of between 50 and 200 μs, typically about100 μs.

[0023] Where there is a single HV pulse, it is preferably a squared pulse. In case of several HV pulses, use can be made of unipolar or bipolar pulses, or of pulses having different directions and/or polarities, preferably of the squared type.

[0024] The HV and LV pulses may be separated by lag and this lag can advantageously be between 300 ms and 3000 s, preferably between 500 ms and 1000 s, typically about 1000 ms.

[0025] In a particular embodiment, there is no lag or only a short one, say less than 300 ms, and the HV pulse has a field strength of between 300 and 1000 volts/cm, preferably of between 400 and 800 volts/cm.

[0026] The nucleic acid is useful in gene therapy, either through expression of a molecule of interest or through modulation or blocking of a gene within the host that have a therapeutic effect. Preferably, the aims of transfection according to the invention are:

- making the muscle a secretory organ for molecules that have a direct or indirect therapeutic effect, including an immunostimulating or vaccinal effect,
- correcting tissue cell, in particular muscle cell, dysfunction.

[0027] In a preferred aspect, the nucleic acid comprises nucleic acid sequences able to express *in vivo* in the transfected tissue cells one or more therapeutically active molecule(s), preferably a protein or proteins of interest. This active molecule may be therapeutically active by itself or indirectly e.g. through a metabolite of said molecule. It may acts in the tissue itself and/or outside the tissue in another location within the body, for example on a tumour located anywhere in the body if the expressed molecule is active against a tumour. As examples of therapeutic molecules of interest, one may refer to those listed in WO-A-99/01158. It will be appreciated that there is no limitation to the kind of molecules that can be expressed in accordance with the invention and therefore the one skilled in the art will be able to carry out the invention with a molecule of interest knowing the coding sequence thereof and routine experimentation to select the best construction or expression vector.

[0028] Any nucleic acid can be used, for example, plasmid DNA, linear DNA, antisense DNA and RNA. In a preferred embodiment, the nucleic acid is a DNA expression vector of the type well known in the art. Generally, an expression vector contains a promoter operably linked to a DNA sequence that codes for the protein of interest, followed by a termination signal such as a polyadenylation signal.

[0029] It will be appreciated that the use according to the invention encompasses the case where two or more nucleic acids able to express *in vivo* different active molecules are used to prepare the medicament. The nucleic acids are preferably chosen so as to be complementary and/or act in a synergistic way in treating a condition.

[0030] Also, is encompassed the use of at least one nucleic acid that is able to express in *vivo* at least two active molecules, that preferably are complementary and/or act in a synergistic way in treating a condition. In that case, the nucleotide sequences encoding the different molecules may be under the control of the same promoter or different promoters.

[0031] According to various aspects of the invention the nucleic acid expresses one or several (at least 2) active molecule(s) selected so that:

- the medicament is efficient in reducing, suppressing or regressing tumor angiogenesis,

- the medicament reduces or suppress tumor growth,
- the medicament inhibits metastasis,
- the medicament is against cancer.

[0032] One embodiment is to transfect tissue, in particular muscle, cells with a construct comprising the Recombinant human Desintegrin Domain of ADAM-15 gene (RDD gene). This gene, its sequence and useful constructs (expression vector pBi-RDD) have been fully described in the article by Trochon-Joseph V. et al. 2004 to which the one skilled in the art may refer. The RDD gene and protein sequences are shown in SEQ ID No.1 and SEQ ID NO.2, respectively. RDD may act as an anti-cancer agent, may reduce or suppress tumor growth, and/or acts as an antiangiogenic and/or antimetastatic agent.

[0033] A specific aspect of the invention is thus the use of a nucleic acid encoding the RDD protein or an efficient fragment thereof (efficient means the protein encoded by the fragment elicits the same or a similar therapeutic activity than the whole RDD polypeptide) for the preparation of a medicament intended to be transferred *in vivo* into tissue cells and to produce therein a RDD polypeptide or a fragment thereof that is therapeutically active, wherein the medicament is injected into a tissue and the tissue is electrically stimulated as follows:

- first with at least one pulse of a High Voltage (HV) field strength of between 200 and 2000 volts/cm
- second with a single pulse of Low Voltage (LV) field strength of between 50 and 140 volts/cm and of duration of between 300 and 2000 ms.

According to an embodiment, the tissue is a muscle. It is preferred that said muscle be electrically stimulated first with at least one pulse of a HV field strength of between 200 and 1400 volts/cm.

[0034] The various characteristics and aspects described *supra* do apply in the same way to this specific use and reference is thus made to the above in this respect in order to further characterise this specific use. This medicament is advantageously useful as an antiangiogenic and/or antimetastatic agent.

[0035] In another interesting aspect, as a therapeutically active molecule, the nucleic acid encodes one or several immunogens (or immunogenic peptides, polypeptides or proteins) that are able to induce an immune response in the host, and preferably a protective immune response. In this aspect, the invention relates to producing an immunogenic composition or a vaccine or a therapeutic vaccine, that is directed against a microorganism, e.g. virus or bacteria, or against cancers. By way of example only, the nucleic acid encodes one or several (at least 2) immunogens of HIV, HBV, Epstein-Barr virus, pseudorabies virus, syncitia forming virus.

[0036] Another object of the present invention is a method of treatment of a Human or an animal, comprising injecting a nucleic acid into a tissue, and electrically stimulating the tissue as follows:

- first with at least one pulse of a High Voltage (HV) field strength of between 200 and 2000 volts/cm
- second with a single pulse of Low Voltage (LV) field strength of between 50 and 140 volts/cm and of duration of between 300 and 2000 ms,

the nucleic acid being transferred into the tissue cells by result of this electric stimulation.

[0037] According to an embodiment, the tissue is a muscle. It is preferred that said muscle be electrically stimulated first with at least one pulse of a HV field strength of between 200 and 1400 volts/cm.

[0038] As described *supra,* according to a preferred aspect, the nucleic acid is able once transferred *in vivo* into tissue cells to produce therein a therapeutically active molecule, that is intended to exert directly or indirectly a therapeutic action in the muscle cells and/or at another body location.

[0039] Preferably, the nucleic acid is injected before applying the single LV pulse and still more preferably, before the application of the HV pulse or pulses. Typically, the injection occurs from a few seconds to 10 minutes, preferably 5 to 10 minutes before the HV pulse or pulses.

[0040] The various characteristics and aspects described *supra* in relation with the use according to the invention do apply in the same way to the method of treatment and reference is thus made to the above in order to further characterize this method.

[0041] One aspect is such a method wherein the nucleic acid encodes the RDD gene or an efficient fragment thereof, as disclosed therein, and the method is intended to reduce or suppress tumor growth, and/or acts as an antiangiogenic and/or antimetastatic agent.

[0042] Still another object of the invention is the electroporation method itself, comprising placing electrodes near a tissue containing a nucleic acid interstitially, then electrically stimulating the tissue as follows:

- first with at least one pulse of a High Voltage (HV) field strength of between 200 and 2000 volts/cm
- second with a single pulse of Low Voltage (LV) field strength of between 50 and 140 volts/cm and of duration of

between 300 and 2000 ms,

the nucleic acid being transferred into the tissue cells by result of this electric stimulation.

[0043] According to an embodiment, the tissue is a muscle. It is preferred that said muscle be electrically stimulated first with at least one pulse of a HV field strength of between 200 and 1400 volts/cm.

[0044] The nucleic acid is heterogeneous to the body and is of the type described supra. It is preferably a nucleic acid comprising nucleic acid sequences able to express *in vivo* in the transfected muscle cells one or more therapeutically active molecule(s), preferably a protein or proteins of interest.

[0045] In an aspect, the electrodes are placed at the contact of the skin, i.e. outside the body and this does not need any surgery act.

[0046] In another aspect, the electrodes are placed at the contact of the tissue, in particular the muscle, itself. In that case, the electrodes may be carried by a device making both the injection of the nucleic acid and the electric stimulation. The electrodes may also be separate from the injection device.

[0047] The various characteristics and aspects described *supra* in relation with the electrotransfer characteristics and the composition of the nucleic acid do apply in the same way to the electroporation method and reference is thus made to the above in order to further characterize this method.

[0048] The present invention will now be described in further details with the presentation of the following non-limitative experiments and with reference to the drawings in which:

Fig. 1. Luciferase expression after DNA electrotransfer using combinations of one or eight HV pulses (800 V/cm; 0.1, 0.2 or 0.5 ms) and four LV pulses (80 V/cm; 100 ms) (xHV+4LV pulse combination). Data are presented as mean ± SD. Statistical difference between each of the xHV+4LV groups was calculated using t-tests; NS - not significant.

Fig. 2. Luciferase expression after DNA electrotransfer using combination of one HV pulse (800 V/cm; 100 μs) and various number of LV pulses (100 ms; 80 V/cm) (HV+xLV pulse combinations). Data are presented as mean ± SD. Statistical difference between neighbor groups shown in the figure was calculated using t-tests and is indicated by asterisks (** P<0.01; *** P<0.001; NS - not significant).

Fig. 3. Luciferase expression after DNA electrotransfer using combination of one HV pulse (800 V/cm; 100 μs) and various number of LV pulses (50 ms; 80 V/cm) (HV+xLV pulse combinations). Data are presented as mean ± SD. Statistical difference between neighbor groups shown in the figure was calculated using t-tests and is indicated by asterisks (* P<0.05; ** P<0.01; NS - not significant).

Fig. 4. Luciferase expression after DNA electrotransfer using combination of one HV pulse (800 V/cm; 100 μs) and LV pulse(s) as a function of pulse number and pulse duration of LV pulse(s) keeping constant the total duration of the LV. Data are presented as mean ± SD. Statistical difference between the 1HV+1LV (400 ms) group and each of the other groups was calculated using t-tests and is indicated by asterisks (* P<0.05; ** P<0.01; *** P<0.001).

Fig. 5. Metastases number in the mice after electotransfer of pBi (control) or pBi-RDD.

EXEMPLE 1

- Materials and Methods

Plasmid DNA

[0049] The plasmid pXL 3031 (pCMV-Luc+) containing the cytomegalovirus promoter (nucleotides 229-890 of pcDNA3, Invitrogen) inserted upstream of the coding sequence of the modified cytosolic luc+ gene coding for the firefly luciferase (Soubrier *et al.,* 1999) was used. The plasmid DNA was prepared using usual procedures (Ausubel *et al.,* 1994). Alternatively, the pEGFP-N1 plasmid (BD Biosciences Clontech, Saint Quentin Yvelines, France) featuring the gene of the Green Fluorescent Protein (GFP) under the control of the CMV promoter and prepared in PBS (phosphate buffered saline, Gibco, Cergy-Pontoise, France) using the EndoFree Plasmid Giga Kit (QIAGEN, Courtabeuf, France) was also used.

Animals

[0050] For all experimental procedures female, 7-9 weeks old, C57Bl/6 mice were anesthetized by the intraperitoneal

administration of the anesthetics Ketamine (100 mg/kg; Ketalar, Panpharma, France) and Xylazine (40 mg/kg; Rompun, Bayer, France). Prior to the experiments the legs were shaved using an electric shaver. At least 10 muscles (5 mice) were included in each experimental group for luciferase determinations. In the case of the GFP qualitative data, four muscles were used for each experimental condition.

DNA injection

[0051] For the luciferase experiments, 3 μg of plasmid DNA prepared in 30 μl of 0.9 % NaCl were injected. In most of the experiments (Figs. 1 to 5), the DNA solution was supplemented with 120 IU/ml heparin (Laboratoires Leo, Saint Quentin en Yvelines, France; one mg of the heparin (MW 10-12 kDa) corresponded to approximately 137 IU). The DNA was injected into *tibial cranial* muscles using a Hamilton syringe with a 26-gauge needle. For GFP experiments, 4 μg in 20 μl of PBS were injected in each treated tibialis, always in the absence of heparin.

DNA electrotransfer

[0052] HV and LV pulse combinations were generated by a device consisting of square wave electropulsator PS-15 (Jouan, St Herblain, France) and a microprocessor-driven switch/function generator built at the University of Ljubljana, Faculty of Electrical Engineering, Slovenia. The device allowed for precise control of every electrical parameter in HV+LV combinations of pulses (Satkauskas *et al.,* 2002).

[0053] HV and LV pulse combinations were delivered soon (40±15 s) after intramuscular DNA injection. In all the experiments the lag between HV and LV was fixed to 1 s. For pulse delivery to the muscles stainless plate electrodes 4.4 mm apart were used. The 1-cm plates encompassed the whole leg of the mice. To ensure good contact between the *tibial cranial* muscle of exposed leg and the plates of the electrodes a conductive gel was used. Electric field values (in V/cm) are always expressed in terms of the ratio of the voltage applied (V) to the distance between the electrodes (cm).

[0054] For the GFP experiments the pulses combinations were delivered using a CLINIPORATOR™ (IGEA, s. r. l., Carpi (MO), Italy) generator and 5 mm apart electrodes from the same company.

Luciferase activity measurement

[0055] The mice were sacrificed 2 days after DNA electrotransfer. The muscles (net weight approximately 60 mg) were took off and homogenized in 1 ml Cell Culture Lysis reagent solution (10 ml Cell Culture Lysis reagent (Promega Charbonnières, France), diluted with 40 ml distilled water and supplemented with 1 tablet of the Protease inhibitor cocktail from Boehringer Mannheim, Mannheim, Germany). After centrifugation at 12,000 rpm for 10 min at 4°C, the luciferase activity was assessed on 10 μl of the supernatant, using a Walac Victor^2 luminometer, by integration of the light produced during 1 s, starting after the addition of 50 μl of Luciferase Assay Substrate (Promega) to the muscle lysate. The results were collected from the luminometer in relative light units (RLU). Calibration with purified firefly luciferase protein showed that 10^6 of RLU correspond to approximately 70 ng of expressed luciferase. The final results were expressed as pg of luciferase per muscle.

GFP fluorescence observations

[0056] The mice were sacrificed 3 days after the injection of the pEGFP-N1 plasmid and the transfected tissue was observed using a Leica MZ12 fluorescence stereomicroscope with a Leica GFP Plus filter set (Art. No. 10446143: excitation filter 480/40 nm, dichroic mirror 505 nm LP, barrier filter 510 nm LP) (Leica, Rueil-Malmaison, France). Pictures were taken using a digital cooled color camera (AxioCam HRc, Zeiss, Le Pecq, France), and the quantification of the GFP expression was made by software (AxioVision Light Edition Release 4.1.1.0) integration of the light detected by the camera.

Statistical analysis

[0057] For statistical comparison of several groups use was made of two-tailed Student's *t*-test for unpaired values. In the figures luciferase expression data was reported as mean ± SD.

Results

[0058] In the case of the luciferase experiments, due to high sensitivity of the measurements, a solution of plasmid DNA supplemented with low amounts of heparin (120 IU/ml) was injected. Heparin at this dose causes a large decrease in the spontaneous uptake of DNA by the muscle but does not significantly impair the efficacy of DNA electrotransfer

into the muscle fibers (Satkauskas *et al.,* 2001). Therefore, the respective contributions of HV and LV pulses on the efficiency of DNA electrotransfer can be analyzed more precisely in the presence of heparin. Additionally, the lag between HV and LV pulse(s) was fixed to 1 s.

Influence of HV pulse duration and number

[0059] To analyze the role of the electropermeabilizing (HV) pulses the LV pulses giving the best level of gene expression according to previous data (Satkauskas et *al.,* 2002) were used. In accordance with this teaching, the LV component parameters were fixed for this experiment to four LVs of 80 V/cm and 100 ms duration, with a delay between the pulses of 1 second.

[0060] Improvement of muscle permeabilization was tried through the increase of either the number of HV pulses (from 1 to 8) or the duration of the HV (from 100 μs to 500 μs). As shown in Fig. 1, neither the increase of HV duration, nor the increase of HV number significantly enhanced muscle transfection.

Influence of LV pulse number

[0061] As a consequence of the results shown in Fig. 1, one single HV of 800 V/cm and 100 μs was always used to analyze the role of the LV component. First, the influence of the number of LVs was examined. The LV pulse strength was fixed to 80 V/cm, duration to 100 ms and the delay between LVs to 1 s. Luciferase expression markedly increased when LV number was increased from 1 to 4 (Fig. 2). Consistently with previous data (Satkauskas *et al.,* 2002), with four LVs the luciferase expression was 10 times higher than with one LV. No further significant increase was observed with a larger number (6 or 8) of LV pulses (Fig. 2).

[0062] Subsequent experiments on the influence of pulse number on gene transfer efficacy were performed using LV (s) of 50 ms duration (Fig. 3). The same trend as in the case of the LV(s) of 100 ms duration (Fig. 2) was observed. In both cases the beginning of the plateau in luciferase gene expression started at a total pulse duration of 400 ms. Again, no further significant increase was observed with increased number (12 or 16) of LV pulses.

[0063] Four different combinations of number and duration of the LVs were further used and compared, all of them resulting in a total duration of the low voltage pulses equal to 400 ms (Fig. 4). A tendency to a progressive decrease in luciferase gene expression with the concomitant decrease in individual pulse duration and increase in pulse number was found (Fig. 4). Remarquably and unexpectedly, HV and LV combinations using a single LV of 400 ms resulted a further increase of and to the best luciferase gene expression, for example about 2 times higher than using eight LVs of 50 ms ($p<0.001$).

GFP fluorescence observations

[0064] After the electrotransfer of the GFP gene using one HV of 100 μs and 800 V/cm followed after 1 sec delay by one 400 ms LV pulse of either 60, 80 or 100 V/cm, the distribution and the intensity of the fluorescence within the muscles were qualitatively and semi-quantitatively measured using a fluorescence stereo microscope. Pictures were taken either at a constant exposure time (100 ms, Panels A, B and C) or at a variable exposure time, i.e. allowing the camera to adjust the exposure time to acquire an equivalent amount of light from picture to picture (Panels D, E and F). The pictures that have been made represent the images observed in four muscles for each experimental condition. Two series of pictures ere made showing the reproducibility of the results as well as the large increase in fluorescence with the increase in the field strength of the LV pulses (Panels A, B and C). The quantitative analysis of the mean density of the green color in these images sustain the qualitative data: in a relative scale with 256 levels of intensity, the levels 41 (left muscle) and 33 (right muscle) were reached at 60 V/cm (panel A), while levels 111 and 89 were reached at 80 V/cm (panel B) and 138 and 127 at 100 V/cm(panel C). These pictures have also shown that the fluorescent 'optical surface' is identical whatever the LV field strength (Panels D, E and F). This increase in fluorescence results from a larger fluorescence of each fiber, but the volume of tissue affected by the electrotransfer was the same. The increase in the number of plasmid molecules electrotransferred into the fibers explains the observed increase in the fluorescence of the individual fibers.

[0065] J'ai modifié le texte de cet exemple pour nous permettre de nous passer de la figure; il est en effet difficile d'utiliser des photographies dans les brevets, la qualité s'ammenuisant au fur et à mesure des copies; il ne me smble pas ici indispensable de les conserver

EXEMPLE 2

[0066] The expression vectors used in this experiment were prepared in accordance with Trochon-Joseph V. et al. 2004.

[0067] 20 μg each of pBi (control), or pBi-RDD (experimental treatment), together with 10 μg of the Tet-tTS and 20 μg of the Tet-On plasmid, in sterile 0.9% NaCl (final volume, 30 μl) were injected into *Tibialis cranialis* muscles. Two

legs per animals underwent electrotransfer.

Electrotransfer was conducted as described below.

[0068] Legs of C57BL/6 mice were shaved using an electric shaver on the day before electrotransfer. Before the electrotransfer procedure, animals were anesthetized via the intraperitoneal injection of a mixture of ketamine (100mg/kg body weight) and xylazine (40mg/kg).

[0069] Plasmid mixture was injected using a Hamilton syringe. A conductive gel was applied to ensure good contact between the leg skin and the two stainless steel plate electrodes (space between the electrodes: 5 mm). Subsequently, one transcutaneous square-wave electric pulse of 700V/cm and 100 μs (1 Hz) was first applied to permeabilize membrane. After a 1000 ms pause and without moving electrodes, one transcutaneous square-wave electric pulse of 100V/cm and 400 ms was applied to allow DNA entry into cells by electrophoretic migration. Electrotransfer was performed with the electropulsator Cliniporator (IGEA, Italy). The same procedure was followed for each animal group and each leg. 10 mice were used in each group.

[0070] Production of RDD from electrotransferred muscles was induced by adding doxycycline into animal drinking water 3 days before tumor implantation. Doxycycline induction was maintained during the experiment.

[0071] Log-phase cultured B16F10 melanoma cells were detached with 0.02% EDTA and resuspended to the final concentration of 2 x 10^6/ml in sterile 0.9% NaCI. 100 μl of the suspension were injected I.V. into the retro-orbital sinus of mice. Seven days later cell injection, mice were sacrificed, the lungs were excised, and metastatic nodules were counted under a dissecting microscope.

[0072] As shown in figure 5, in presence of RDD, 70.5% fewer metastatic nodules were detected in the experimental group than in controls. RDD inhibited the development of B16F10 melanoma.

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SEQUENCE LISTING

<110> Bioalliance Pharma
Centre National de la Recherche Scientifique
Institut Gustave Roussy

<120> Electrotransfer of nucleic acid into tissue cells

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Arg Gly Asp Cys Asp Leu Pro Glu Phe Cys Pro Gly Asp Ser Ser Gln
65                  70                  75                  80

Cys Pro Pro Asp Val Ser Leu Gly Asp Gly Glu
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Claims

1. Use of a nucleic acid for the preparation of a medicament intended to be transferred *in vivo* into tissue cells, wherein the medicament is brought into contact with tissue cells and the tissue is electrically stimulated as follows:

   - first with at least one pulse of a High Voltage field strength of between 200 and 2000 volts/cm
   - second with a single pulse of Low Voltage field strength of between 50 and 140 volts/cm and of duration of between 300 and 2000 ms.

2. The use according to claim 1, wherein the single pulse of Low Voltage has a field strength of between 80 and 120 volts/cm.

3. The use according to claim 1, wherein the single pulse of Low Voltage has a field strength of between 90 and 110 volts/cm.

4. The use according to claim 1, wherein the single pulse of Low Voltage has a field strength of 100 volts/cm.

5. The use according to any one of claims 1 to 4, wherein the single pulse of Low Voltage has a duration of between 300 and 800 ms.

6. The use according to claim 5, wherein the single pulse of Low Voltage has a duration of between 350 and 600 ms.

7. The use according to claim 5, wherein the single pulse of Low Voltage has a duration of 400 ms.

8. The use according to any one of claims 1 to 7, wherein the single pulse of Low Voltage has a polarity which is opposite to that of the High Voltage pulse.

9. The use according to any one of claims 1 to 8, wherein a single High Voltage pulse is used.

10. The use according to any one of claims 1 to 9, wherein use is made of High Voltage field strength of between 200 and 1400 volts/cm.

11. The use according to claim 10, wherein use is made of High Voltage field strength of between 400 and 1200 volts/cm.

12. The use according to claim 10, wherein use is made of High Voltage field strength of between 600 and 800 volts/cm.

13. The use according to claims 10, wherein use is made of High Voltage field strength of 700 volts/cm.

14. The use according to any one of claims 1 to 13, wherein use is made of High Voltage field pulse(s) having a duration of between 10 and 1000 μs.

15. The use according to claim 14, wherein use is made of High Voltage field pulse(s) having a duration of between 50 and 200 μs.

16. The use according to claim 15, wherein use is made of High Voltage field pulse(s) having a duration of 100 μs.

17. The use according to any one of the preceding claims, wherein High Voltage pulse and Low Voltage pulse are separated by a lag.

18. The use according to claim 17, wherein the lag is of between 300 ms and 3000 s.

19. The use according to claim 17, wherein the lag is of between 500 ms and 1000 s.

20. The use according to claim 17, wherein the lag is of 1000 ms.

21. The use according to any one of the preceding claims, wherein the nucleic acid is able to produce a therapeutically active molecule or several therapeutically active molecules in the tissue cells.

22. The use according to any one of the preceding claims, wherein use is made of two or more nucleic acids able to produce different therapeutically active in the tissue cells.

23. The use according to any one of the preceding claims, wherein the nucleic acid comprises the RDD gene.

24. The use according to any one of the preceding claims, wherein the medicament is efficient in reducing or suppressing tumor angiogenesis.

25. The use according to any one of the preceding claims, wherein the medicament reduces or suppresses tumor growth.

26. The use according to any one of the preceding claims, wherein the medicament inhibits metastasis.

27. The use according to any one of the preceding claims, wherein the medicament is against cancer.

28. The use according to any one of claims 1 to 20, wherein the nucleic acid encodes one or several immunogens.

29. The use according to claim 27, wherein the immunogen(s) is/are HIV immunogen(s).

30. The use according to any of claims 1 to 29, wherein said tissue is a muscle.

31. An electroporation method, comprising placing electrodes near a tissue at the contact of the skin, wherein the tissue contains a nucleic acid interstitially, then electrically stimulating the muscle as follows:

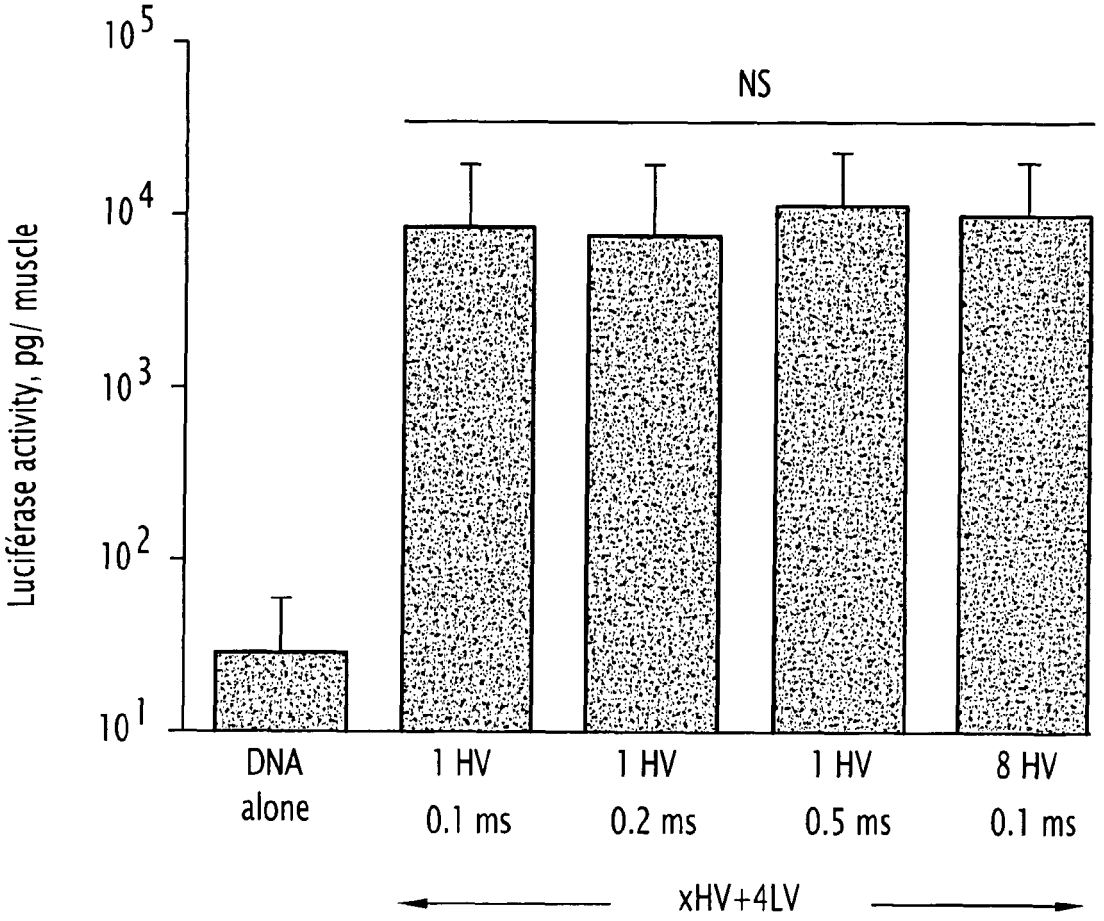
- first with at least one pulse of a High Voltage (HV) field strength of between 200 and 2000 volts/cm
- second with a single pulse of Low Voltage (LV) field strength of between 50 and 140 volts/cm and of duration of between 300 and 2000 ms,

the nucleic acid being transferred into the tissue cells by result of this electric stimulation.

32. The method of claim 31, wherein the stimulation is as defined in any one of claims 2 to 20.

33. The method of claim 31 or 32, wherein the nucleic acid is as defined in claim 21, 22, or 23.

34. The method according to any of claims 31 to 33, wherein said tissue is a muscle.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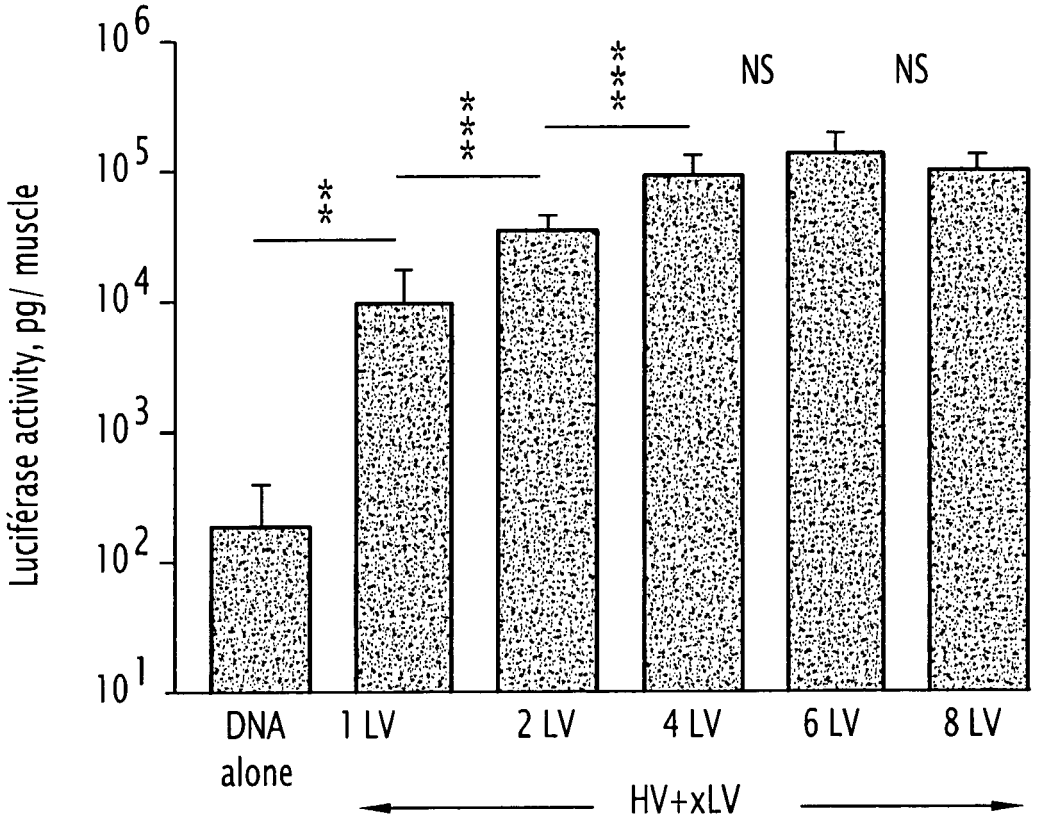
xHV+4LV

FIG.1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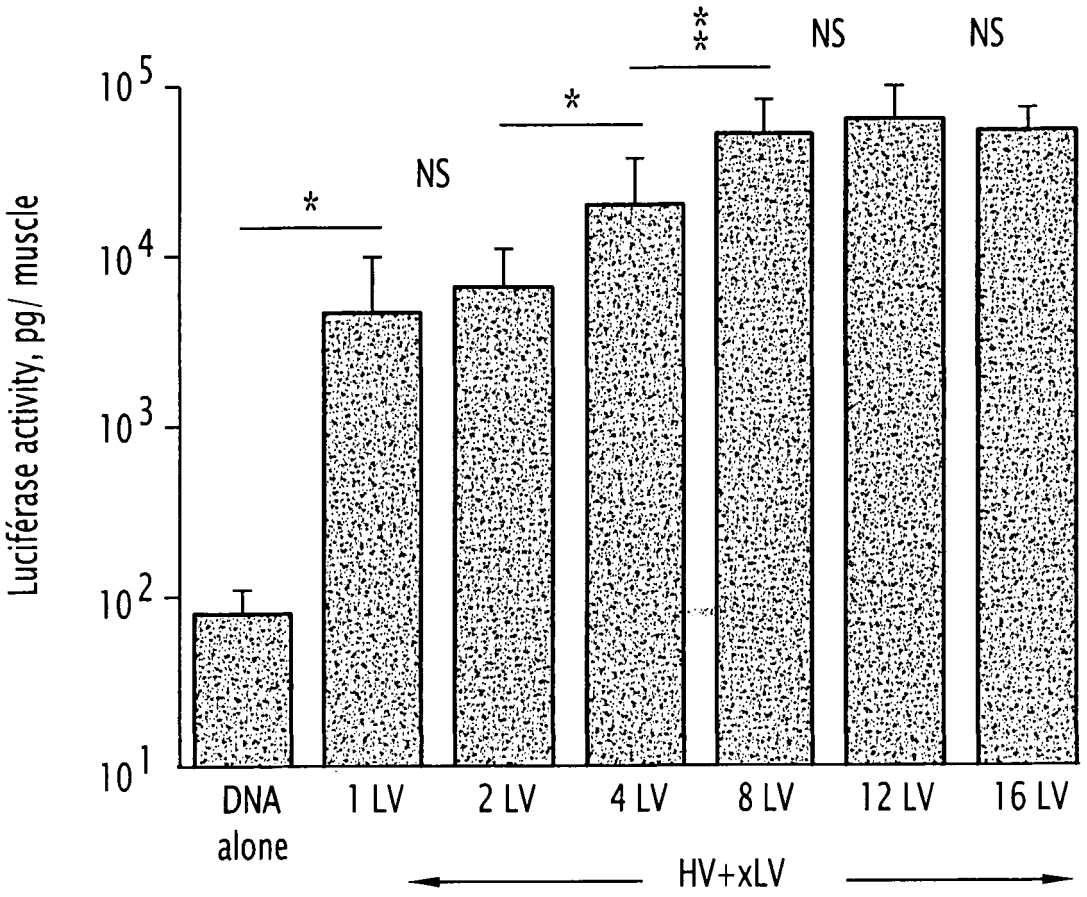
4 LV
6 LV
8 LV
HV+xLV

FIG.2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10^4
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10^2
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DNA alone
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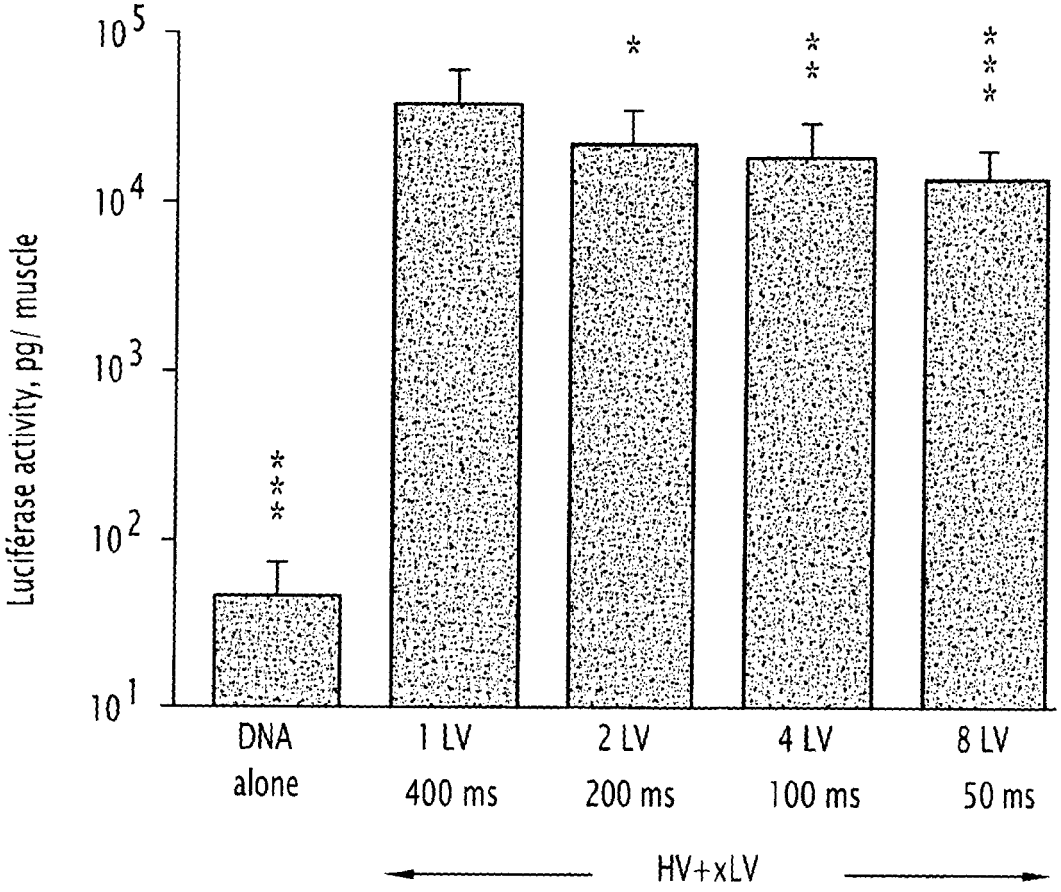
12 LV
16 LV
HV+xLV

FIG.3


Luciférase activity, pg/ muscle
10^5
10^4
10^3
10^2
10^1
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*
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DNA alone
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2 LV 200 ms
4 LV 100 ms
8 LV 50 ms
HV+xLV

FIG.4

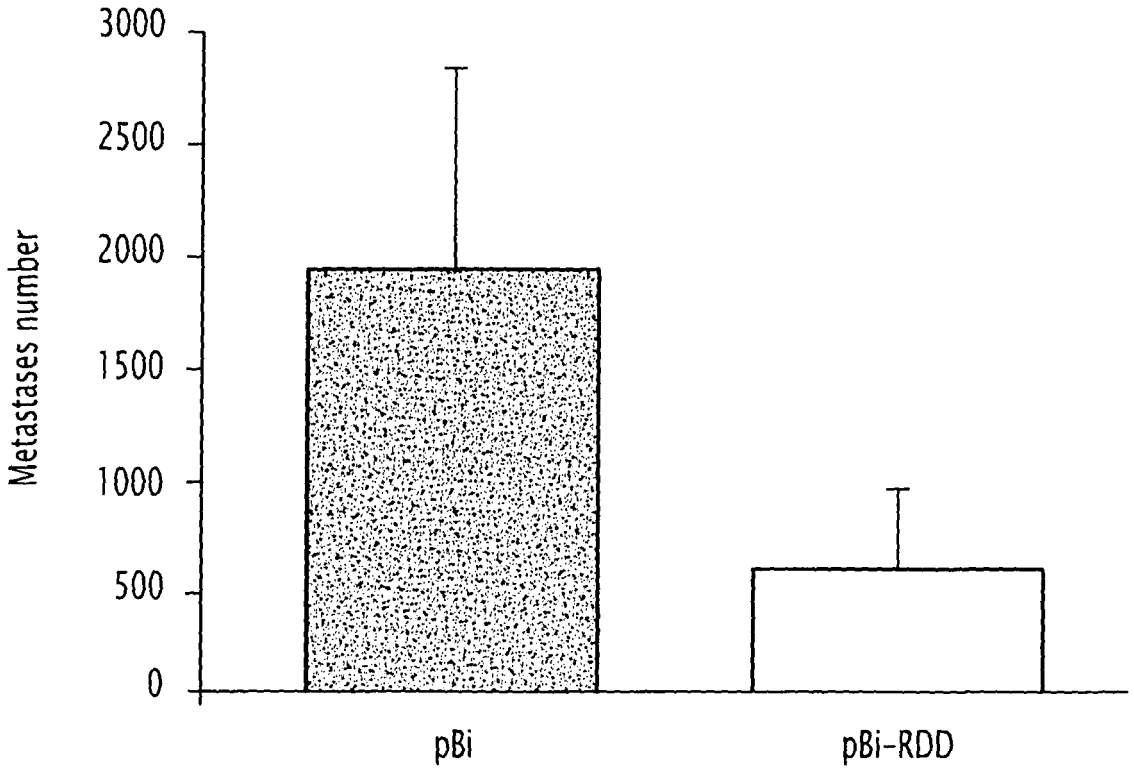

FIG.5

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 05 29 1825

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	26 April 2006	Donath, C

CATEGORY OF CITED DOCUMENTS

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 05 29 1825

DOCUMENTS CONSIDERED TO BE RELEVANT

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Place of search	Date of completion of the search	Examiner
Munich	26 April 2006	Donath, C

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O : non-written disclosure
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26-04-2006

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